

## Review

Status and challenges of filovirus vaccines<sup>☆</sup>Douglas S. Reed<sup>a,\*</sup>, Mansour Mohamadzadeh<sup>b</sup><sup>a</sup> Center for Aerobiological Sciences, U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID),  
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**Abstract**

Vaccines that could protect humans against the highly lethal Marburg and Ebola viruses have eluded scientists for decades. Classical approaches have been generally unsuccessful for Marburg and Ebola viruses and pose enormous safety concerns as well. Modern approaches, in particular those using vector-based approaches have met with success in nonhuman primate models although success against Ebola has been more difficult to achieve than Marburg. Despite these successes, more work remains to be done. For the vector-based vaccines, safety in humans and potency in the face of pre-existing anti-vector immunity may be critical thresholds for licensure. The immunological mechanism(s) by which these vaccines protect has not yet been convincingly determined. Licensure of these vaccines for natural outbreaks may be possible through clinical trials although this will be very difficult; licensure may also be possible by pivotal efficacy studies in animal models with an appropriate challenge. Nevertheless, nonhuman primate studies have shown that protection against Marburg and Ebola is possible and there is hope that one day a vaccine will be licensed for human use.

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## 1. Introduction

The viruses that comprise the family Filoviridae cause some of the most lethal viral hemorrhagic fevers known. In 1967, an outbreak in Marburg, Germany occurred among laboratory personnel that handled monkeys or tissues subsequently determined to be infected with a small, unidentified, and negative-strand RNA virus [1,2]. With a case-fatality rate of 22% and an unknown route of transmission, there was considerable concern about Marburg virus (MARV) (now termed *Lake Victoria marburgvirus*); however, there were only a limited number of secondary cases. In 1976, a MARV-like virus emerged in two nearly simultaneous outbreaks in Africa along the Ebola River; the case-fatality rates, however, were substantially higher (50–80%) than in the MARV outbreak. Two distinct viruses were isolated from these outbreaks, *Zaire ebolavirus* (ZEBOV) and *Sudan ebolavirus* (SEBOV), their names based on the locations of the initial outbreaks [3,4].

Since 1976, there have been sporadic cases and outbreaks in Africa of ZEBOV, SEBOV, and MARV. Two other strains of Ebola virus have been identified, Cote d'Ivoire (CIEBOV) and Reston (REBOV) [5,6]. While CIEBOV and REBOV are highly pathogenic in nonhuman primates, only one human case of CIEBOV has been reported, and it is not clear whether REBOV is virulent in humans. Until recently, ZEBOV was thought to be the most virulent of all filoviruses, with case-fatality rates around 80%, while SEBOV was slightly less pathogenic with case-fatality rates around 50%. Recent outbreaks in the Congo and Angola have demonstrated that MARV strains can be as virulent as ZEBOV [7,8].

The number of cases in these outbreaks has generally been small and implementation of general barrier-nursing procedures appears to bring a halt to these outbreaks. However, there is still considerable concern about these viruses and much that is not known. No licensed vaccines or therapeutic

tics exist that can offer protection against these viruses, so they can only be handled in biosafety level-4 (BSL-4) laboratories. Recent data suggest bats may be a host [9] but even if bats are proven to be the sole host species for all filoviruses, control of outbreaks in Africa may be exceedingly difficult. Epidemics among chimpanzees and great apes have occurred, with potentially catastrophic effects on the populations of these endangered animals [10–12]. In the last decade, the number of outbreaks for both EBOV and MARV viruses in Africa has risen, leading to concerns that it is only a matter of time before cases are seen in a developed nation. Of paramount concern are the assertions that the former Soviet Union considered using filoviruses as offensive biological weapons and may have weaponized MARV for aerosol dissemination [13].

The high mortality rates seen with filovirus outbreaks and the knowledge that these viruses could be employed as biological weapons are the primary reasons these viruses are listed as Category A Priority Pathogens by the National Institutes of Health [14]. Licensed vaccines and therapeutics that can protect against aerosol exposure to either MARV or EBOV are needed to protect against this threat.

## 2. The Filoviridae

The genomes of all filoviruses are composed of a non-segmented, negative sense, single-strand RNA approximately 19-kb long, encoding genes for NP (major nucleoprotein), VP35 (P-like protein), VP40 (matrix protein), GP (glycoprotein), VP30 (minor nucleoprotein), and VP24 and L (RNA-dependent RNA polymerase). The known transcribed open reading frames of the viral genes, gene order, and presumptive protein functions are shown in Fig. 1. Expression of VP40 in combination with GP is sufficient to generate virus-like particles of MARV and EBOV that resemble infec-

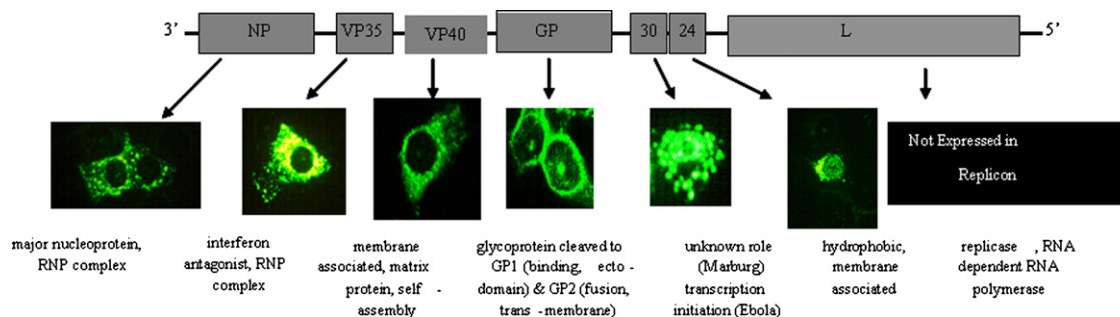


Fig. 1. Genome of Marburg virus and expression in cells.

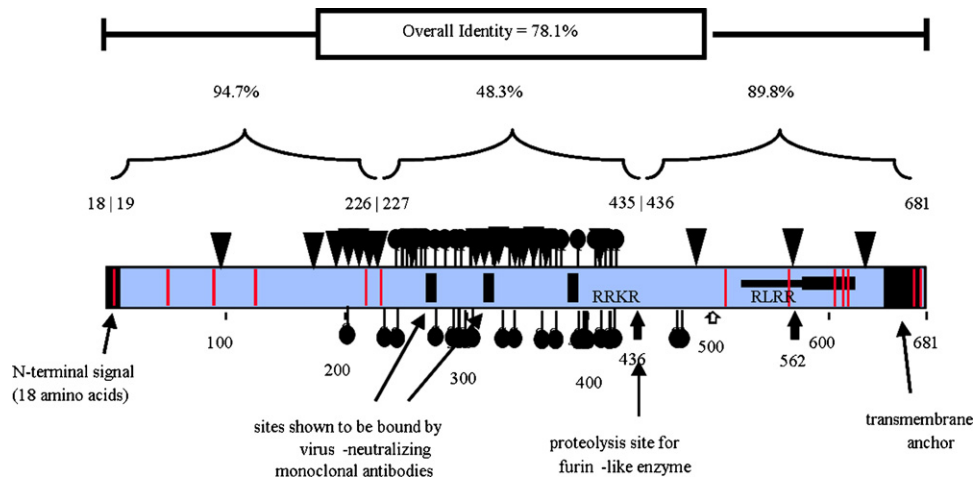


Fig. 2. Genetic divergence among different strains of Marburg virus.

tious virus in morphology [15]. VP40 and GP share sufficient homology between different filoviruses that heterologous virus-like particles can be generated which express the VP40 of one virus and the GP of another [16].

Differences in GP tend to cluster in the central portion of the gene, a region which, when translated and proteolytically processed, becomes the highly glycosylated, distal portion of trimeric GP spikes [17]. Significantly, this “variable” region of GP is highly immunogenic as determined in part by monoclonal antibodies obtained against MARV and EBOV GP [18–20]. GP from both MARV and EBOV have been implicated in the pathogenesis of these infections including an increase in vascular permeability [17,21]. Transcriptional editing of GP results in multiple forms of GP including two transmembrane forms (GP<sub>1</sub> and GP<sub>1,2</sub>); although all filovirus-infected cells secrete GP, ZEBOV produces an additional secreted form and small  $\Delta$  peptide [22]. The role of secreted GP (particularly the unique forms produced by ZEBOV) is unclear at this time although it has been postulated to play a role in evasion or suppression of the immune response.

As mentioned above, EBOV are divided into four distinct species—ZEBOV, SEBOV, CIEBOV, and REBOV that are different not only genetically and antigenically but also in terms of pathogenesis and virulence. All ZEBOV strains to date cause lethal disease even at exceedingly low doses in macaques; the virulence of SEBOV in NHP is strain dependent (Tom Geisbert, personal communication). REBOV is highly pathogenic in macaques but does not appear to cause disease in African green monkeys or humans [6,23,24]. CIEBOV is clearly pathogenic for chimpanzees, however, the only known human case survived exposure leading to speculation that it is less virulent than ZEBOV and SEBOV [5,12,25]. CIEBOV has not been as well studied experimentally as the other EBOV species.

Taxonomically, MARV is considered a single viral species; however, MARV includes a constellation of viruses that differ genetically, antigenically, and phenotypically. The

Russian literature centers upon an isolate called Popp [26]. Work at USAMRIID has focused on the Musoke [27], Ravn [28], and Ci67 [1] isolates. Ci67 is identical to Popp in the amino acid sequence of its glycoprotein (GP) (L. Lofts, A. Schmaljohn, personal communication). Both Popp and Ci67 arose from the first MARV outbreak in Germany and Yugoslavia in 1967. In the amino acid sequence of the GP protein, the 1967 viruses differ from Musoke by 7% and from the most divergent isolate, Ravn, by 22% (Fig. 2) [28]. Ci67 grows more rapidly in vitro and forms larger plaques on Vero E6 cells than Musoke or Ravn. More importantly, Ci67 produces an almost 10-fold higher viral burden in naive cynomolgus macaques at the late stages of disease (A. Schmaljohn, unpublished data). The more recently identified MARV isolate from the 2005 Angola outbreak differs from the Popp and Musoke strains by approximately 7% [29].

### 3. Human disease

In humans, MARV and EBOV incubation periods range from 2 to 14 days. Typical presentation is an acute, unremarkable febrile illness with symptoms including chills, headache, and myalgia [30]. Mental confusion or changes in personality have been reported, particularly with MARV. Nausea, vomiting, abdominal pain, diarrhea, sore throat, and a maculopapular rash have been reported in some but not all cases. Within 6–8 days of fever, hemorrhagic complications can develop, and patients develop increasingly severe symptoms including severe weight loss, delirium, shock, liver failure, massive hemorrhaging, and multi-organ dysfunction. For ZEBOV, clinical signs include elevated liver enzymes, pronounced decreases in peripheral blood lymphocyte counts, especially CD8 T cells, and elevations in inflammatory cytokines [31–34]. Thrombocytopenia is reported as well as decreases in platelet counts and development of disseminated intravascular coagulation in some but not all fatal cases. Patients that survive have a prolonged convalescence marked

by fatigue and can continue to harbor infectious virus for months after recovery [2,35,36].

There are differences in the diseases caused by different strains of MARV and EBOV, for reasons that are not well understood. Neurological complications are more commonly reported with MARV infection than with EBOV. Immunosuppression and lymphocyte apoptosis are thought to be more profound with ZEBOV infection than the other filoviruses. As mentioned above, CIEBVO and REBOV may be significantly less virulent in humans than SEBOV or ZEBOV while retaining considerable lethality in NHP. At one time MARV was thought to be the ‘lesser’ cousin of EBOV, due to the fact that in the only emergence prior to 1999 (the outbreak in 1967) the mortality rate was ‘only’ 22%. In 1999 an outbreak of MARV associated with a gold mine in the Democratic Republic of the Congo had a mortality rate of >90%; subsequent analyses turned up evidence for multiple strains of MARV causing individual cases [37]. In 2005, however, a new strain of MARV emerged in Angola with a mortality rate of 89% [8]. Preliminary studies in NHP suggests that MARV Angola is just as virulent as ZEBOV [38].

#### 4. Animal models of the human disease

Since the first known outbreak of MARV, animal models have been critical to the study of filoviruses. Both rodent and nonhuman primate (NHP) models exist for MARV and EBOV [39]. Because the number of human cases is low and the availability of human tissues from fatal cases is limited, animal models have been used to study the underlying pathology of the diseases caused by MARV and EBOV. Most of these studies have focused on infection of the viruses by i.p., s.c., or i.m. injections although a limited number of studies have examined infection by aerosol exposure or through the ocular conjunctiva [40–46].

Rodent models of filovirus-mediated disease require adaptation by serial passage for uniform lethality; only the guinea pig has been successfully developed for both MARV and EBOV [2,47] although a mouse-adapted strain of ZEBOV [48] has been developed. Because the mouse model does not develop the severe coagulopathy or lymphocyte apoptosis that is seen in the human disease and lethal disease can only be induced by i.p. inoculation of the virus, the mouse model is not considered ideal for studies of the human disease. Mice have, however, been used as a tool for studying innate immunity, screening vaccine candidates, and elucidating protective epitopes for humoral and cellular immunity to ZEBOV [49–58].

Unlike mice, guinea pigs do develop more severe coagulation defects (drop in platelet counts, increase in coagulation time) after infection with either MARV or ZEBOV but the level of fibrin deposition and coagulopathies seen are not at the levels seen in NHP models. To some extent, this may be dependent upon the strain of guinea pig used, the viral strain, the degree of adaptation, or all three [47,59].

Vaccine efficacy in mouse and guinea pig models has not always been predictive of success in NHPs; a review of the data published to date indicates that it is far easier to protect rodents than NHP against either MARV or EBOV. Vaccines that protected guinea pigs against challenge with heterologous strains of MARV failed to protect NHP [60]. However, that should not detract from the utility of rodent models as a screening tool. It would be difficult to argue that a vaccine that failed to protect in a rodent model should be advanced into NHP studies. Guinea pig-adapted strains of MARV and EBOV appear to lose none of their virulence for NHP [61–63]. Two of three NHP infected with mouse-adapted ZEBOV survived despite developing clinical signs of disease and were subsequently protected against challenge with wild-type ZEBOV; although animal numbers are limited this study highlights why safety concerns would prevent attenuated strains of filovirus from serious consideration as vaccine candidates [64].

NHP are the most relevant animal models of the human disease. Several species of NHP have been used for the study of filoviruses. African green monkeys, rhesus macaques, cynomolgus macaques, and baboons have all been used in filovirus studies. All MARV and EBOV strains are highly pathogenic in both cynomolgus and rhesus macaques, while African green monkeys are resistant to REBOV and baboons appear to be somewhat more resistant to all EBOV [24,39,65,66]. Dose, route, viral strain, and species of NHP used all appear to influence the onset, duration, and severity of clinical signs. Each NHP species appears to develop some, but not all, of the clinical signs and pathology relevant to human disease. For example, with ZEBOV infection cynomolgus macaques and rhesus macaques are more likely to develop a petechial rash and coagulation defects while baboons are more likely to develop hemorrhagic complications.

*Estimates* for the lethality of injected MARV and EBOV in NHP suggest the LD<sub>50</sub> for each is quite low, although this has not been formally established. Cynomolgus macaques are thought by some to be the most ‘stringent’ NHP model for ZEBOV, based on the virulence of ZEBOV in cynomolgus macaques compared to other NHP species. For cynomolgus macaques infected with ZEBOV, the onset of clinical signs is fairly rapid (4–5 days) and fever duration is short (2–3 days) before animals are moribund. In other NHP models, fever onset and duration are slower and thus more like what has been observed in humans. The decision as to which NHP species is most ‘appropriate’ or ‘relevant’ is still a matter of debate; there are currently no data available comparing the disease course and pathogenesis in the different NHP species using the same isolate at the same dose by the same route of challenge. Choices of NHP species have tended to revolve around “the possible,” the historically grounded, and the particular needs of the studies. As vaccines advance towards licensure, a more rational selection of which NHP species should be used for ‘pivotal’ efficacy studies will need to be made based on an understanding of the disease and the immune system of each NHP species, bridging that informa-



tion with what we know occurs in humans, and comparative studies including identical challenge conditions and isolates.

Very little is known about the pathogenesis or disease course after aerosol exposure to filoviruses. Aerosol is not thought to be the ordinary route of transmission in natural outbreaks; however, there have been reports of naive NHP infected across the room from experimentally infected NHP and human cases in which direct contact with infected patients could not be demonstrated, suggesting aerosol transmission [60,67,68]. Experimental studies have shown that filoviruses are relatively stable in aerosol, can survive on surfaces for prolonged periods of time, and are able to infect susceptible naive animals and cause lethal disease when inhaled [40–44,61,69,70]. Because there is concern that these viruses could be used as a biological weapon, where the route of exposure would be aerosol, a better understanding of the pathogenesis of the disease in animals after aerosol exposure and how the disease course after aerosol exposure differs from parenteral inoculation is needed.

While licensure for natural outbreaks may be possible through clinical trials in Africa, clinical trials to determine efficacy against aerosol exposure are ethically and logistically impossible. Licensure of vaccines for this indication will only be possible through the Food & Drug Administration's Animal Rule [71]. Key tenets to the Animal Rule include an understanding of the pathological mechanisms involved in the disease, the mechanisms involved in protection against the disease by the vaccine or therapeutic, and an understanding of the relevance of animal model to humans not only in terms of the disease but also the mechanism(s) involved in protection. Licensure under the Animal Rule will be particularly difficult for aerosol exposure as human data are lacking.

## 5. 'Classical' approaches

The earliest attempts to generate filovirus vaccines were based on the classical approach; i.e., inactivated virus. Classical attenuation by passage through cell culture or another species is not considered a viable option; guinea pig-adapted ZEBOV and MARV retain their virulence for NHP [40,61,62,72] and the reversion rate of other attenuated virus vaccines make clinical trials and licensure of such a vaccine for filoviruses extremely improbable. Recombinant genetic engineering to attenuate infectious clones of MARV and EBOV might be possible, as has been done with Venezuelan equine encephalitis [73], however, demonstrating attenuation would be difficult.

One early approach involved the use of MARV inactivated by formalin, which protected outbred guinea pigs against challenge with a relatively low dose of MARV [74]. When MARV inactivated by  $\gamma$ -irradiation was injected into rhesus macaques, however, one of the six vaccinated macaques failed to respond to the vaccine and only three survived when challenged with MARV [75]. Similarly, formalin-inactivated

ZEBOV combined with aluminum hydroxide was able to protect guinea pigs but only poorly protected NHP against s.c. inoculation with ZEBOV [76]. Sucrose gradient-purified,  $\gamma$ -irradiated ZEBOV failed to protect either cynomolgus macaques or rhesus macaques when combined with RIBI adjuvant or liposomes containing lipid A [77]. Immune responses in all three studies were poor, with minimal IgG responses as detected by ELISA and low or nonexistent neutralizing antibody titers. It is noteworthy, however, that very small numbers of animals were used in all of these studies and no attempts were reported regarding optimization of dose, schedule, or adjuvant.

Expression of ZEBOV and MARV GP in a baculovirus expression system has been approached as a means of producing subunit filovirus vaccines. Baculovirus-expressed MARV and ZEBOV GP have different glycosylation patterns than wild-type viruses [78,79]. Baculovirus-expressed MARV GP lacking the transmembrane domain (GP $\Delta$ TM) was able to protect most guinea pigs when given with RIBI adjuvant and fully protected guinea pigs when given as a booster vaccination after a DNA prime [60]. For the guinea pigs vaccinated with MARV GP $\Delta$ TM, endpoint ELISA titers were comparable between survivors and animals that succumbed to the infection and in vitro neutralizing antibody was not detectable before challenge. Neither the baculovirus GP $\Delta$ TM alone nor the combination DNA/baculovirus GP $\Delta$ TM approaches were able to do any better than partially protect guinea pigs challenged with ZEBOV [79].

## 6. 'New' approaches

### 6.1. DNA vaccines

DNA vaccines expressing the GP of MARV and ZEBOV have been evaluated as potential vaccines in rodents. In guinea pigs, a DNA vaccine expressing MARV GP was weakly immunogenic compared to other strategies and offered incomplete protection when given alone but worked well when boosted with baculovirus-expressed GP $\Delta$ TM [60]. In NHP, the DNA MARV GP vaccine protected four of six cynomolgus macaques from lethal MARV infection [80]. In mice, a DNA vaccine expressing GP from ZEBOV was able to fully protect mice against ZEBOV challenge [80] but failed to adequately protect guinea pigs [79]. Another DNA vaccine construct, however, was successful in protecting guinea pigs against ZEBOV challenge [81]. Based on the success of this second construct with an adenovirus boost in NHP (see below), a phase I clinical trial of a ZEBOV DNA vaccine was initiated in 2004 [82].

### 6.2. Vector-based vaccines

Modern molecular biology has made it possible to insert and express genes of interest by using a variety of systems. One approach that received considerable attention with vac-

cines is to use viruses as vectors expressing a heterologous gene of interest. A stronger and more durable immunological response is typically seen with live vaccines due to replication of the pathogen in the host. However, the risk of adverse events is considerably higher and therefore there are heightened concerns regarding the safety of these vaccines. A significant portion of the general population is immunosuppressed and might be susceptible to the vaccine itself (a concern raised with the use of vaccinia for resumption of vaccination against poxviruses). ‘Defective’ viruses are considered to be safer than live viruses as vectors for vaccines; however, considerably more of the vaccine may be required (and in multiple doses) to achieve protective, long-lasting immunity. Another concern with vectored vaccines is pre-existing immunity to the vector; if significant portions of the population have immunity to the vector, this can effectively block the development of immunity to the heterologous proteins of interest.

#### 6.2.1. Virus-like replicon particles (VRP)

The first approach to meet with success (defined as  $\geq 80\%$  protection) in protecting NHP against a filovirus made use of MARV VRP derived from Venezuelan equine encephalitis (VEE) virus [19]. Because VEE virus has a positive-sense RNA genome, full-length cDNA clones of VEE virus can be used to generate RNA transcripts that, when introduced into susceptible cells, will initiate a complete viral replication cycle and generate infectious virus [83]. Genes of interest such as the GP1-GP2 of MARV replace the VEE virus structural protein gene region in a cDNA plasmid of the VEE viral genome; an RNA transcript from such a plasmid, when introduced into cells, will then replicate and express the heterologous genes. Because the RNA transcript does not contain the structural genes of VEE virus, it is single-cycle, propagation-defective RNA and replicates only in the cells to which it is delivered. The RNA is packaged into VRP by supplying the structural protein genes of VEE virus in trans. Only the replicon RNA is packaged into the VRP, as the helper RNAs lack the packaging sequence required for encapsidation. VRP have been used successfully in mice as a tool to examine the T-lymphocyte response to ZEBOV and to generate monoclonal antibodies that protected mice against a ZEBOV challenge [18,54,58].

When guinea pigs or cynomolgus macaques were vaccinated with VRP containing the GP from the Musoke strain of MARV, they were fully protected against subsequent s.c. inoculation with virulent Musoke [78]. In guinea pigs, there was no difference in the protection afforded by a one-, two-, or three-dose regimen. Although ELISA indicated the presence of anti-MARV IgG, no in vitro neutralizing antibody activity was detectable in the macaques before challenge. Guinea pigs vaccinated with Musoke VRP were protected against challenge with heterologous strains of MARV (Ci67, Ravn) [60] but cynomolgus macaques were not (unpublished data). Initial efforts to extend this success to ZEBOV were not unsuccessful in NHP [77]. Given the success in mice

and guinea pigs, the failure of VRP to protect NHP against heterologous MARV or ZEBOV is likely a function of the strength of the immunological response to the vaccination and further optimization of the dose, schedule, route, and vector should overcome this concern.

Three major obstacles lay in the path of licensure for filovirus VRP-based vaccines. First, the difficulty in protecting against heterologous filoviruses will require testing combinations of VRP expressing GP from the heterologous strains of MARV and EBOV. New VRP may have to be generated as new isolates of MARV and EBOV are identified, such as the Angola strain of MARV. Secondly, optimization of vaccine dose and schedule is needed and in humans there may be issues of patient compliance if multiple vaccinations are required to achieve the needed potency. If high doses for multiple immunizations are required, manufacturing of sufficient VRP may be an issue although it should be noted that vaccines using VRP for other diseases are currently in clinical trials [84]. Finally, VEE is also considered a biodefense threat and armed forces and first responders may be vaccinated with VEE vaccine; as a result, pre-existing immunity to VEE may reduce the effectiveness of vaccination with VRP-based vaccines as has been seen with VEE vaccines given to personnel receiving vaccines for other alphaviruses [85].

#### 6.2.2. Adenovirus-based vaccines

In 2000, Sullivan et al. reported the first successful effort ( $\geq 80\%$  protection) to protect nonhuman primates against challenge with ZEBOV [86]. A combination DNA vaccine prime/adenovirus vector boost was able to fully protect cynomolgus macaques when using GP and nucleoprotein (NP) from ZEBOV. Antibody (by ELISA) and cellular immune responses (by  $^3\text{H}$ -thymidine) to GP were detectable before challenge. The proliferative response was entirely by  $\text{CD4}^+$  lymphocytes;  $\text{CD8}^+$  lymphocytes were not a significant component of the proliferative response. However, the challenge dose was considerably lower than what has been postulated for an accidental needlestick exposure, although it was sufficient to be lethal to all four of the control macaques.

In a subsequent study, Sullivan et al. reported that a single vaccination with the adenovirus vector expressing ZEBOV GP was sufficient to fully protect macaques against both low (10 pfu) and high dose (1000 pfu) challenges [87]. Antibody responses were lower than what had been reported with the DNA prime/adenovirus boost. Intracellular cytokine staining detected a low  $\text{CD8}^+$  response in peripheral blood mononuclear cells but was unable to detect a  $\text{CD4}^+$  lymphocyte response before challenge. It is not clear whether the differences in the cellular immune response from the previous study were due to the change in the vaccination regimen or differences in the types of assays employed to measure cellular immune responses.

Two challenges face the adenovirus ZEBOV GP vaccine. The first is dose; because the adenovirus construct used in these studies was replication deficient, a high dose of particles was required for vaccination. In the ‘accelerated’ vaccination

study, a dose of  $2 \times 10^{12}$  adenovirus particles was used; subsequent studies have shown vaccination with  $10^{10}$  was still protective, while  $10^9$  was not [88]. The second and more serious challenge facing the adenovirus-vectored ZEBOV GP vaccine is the issue of pre-existing immunity to the adenovirus vector. Adenovirus vectors were first employed as a means of gene delivery into human patients, but pre-existing immunity to the vector completely blocks expression of the delivered genes [89]. The studies reported by Sullivan et al. used a serotype 5 adenovirus (Ad5); estimates of human populations indicate that potentially as many as 50% of humans have immunity to Ad5. It is not clear whether pre-existing immunity to Ad5 in humans will prevent development of a protective immune response to ZEBOV. There are recent reports in mice and rhesus macaques that pre-existing immunity to Ad5 did diminish the response to Ad5-vectored vaccines [90,91], however, another study in mice using Ad5-vectors have found that pre-existing immunity did not prevent the development of immunity [92]. Priming with DNA plasmids expressing the gene of interest may be sufficient to overcome pre-existing immunity to the viral vector; however, to date this has only been demonstrated in mice [93–95]. Other serotypes that are not as prevalent in the human population (or that do not typically infect humans at all) might be used in place of Ad5 [90] but the original studies would have to be repeated with the new constructs to demonstrate comparable protection.

#### 6.2.3. Vesicular stomatitis virus-based vaccines

Vesicular stomatitis virus (VSV) has also garnered recent interest as a potential vaccine vector [96]. VSV expressing GP of ZEBOV or MARV can elicit complete protection against challenge in cynomolgus macaques. All vaccinated animals were protected, and more significantly were protected against rechallenge with heterologous strains. Antibody responses were detected before challenge by ELISA but in vitro neutralizing antibody and cellular immune responses (as measured by intracellular cytokine staining) were not. VSV-vectored MARV vaccine has also been shown to protect macaques against heterologous MARV viruses and can protect against MARV infection even when given postexposure [97]. VSV-vectored vaccines do not currently face the issues of pre-existing immunity that may plague vaccinia, adenovirus, or VEE-based vaccines. However, as the particular construct used in the VSV studies is a live attenuated virus and not replication deficient, there will be significant safety concerns as VSV can cause human disease. Studies in animals suggest that VSV can become latent and persist for some time in host tissues after convalescence [98,99]. These safety concerns may necessitate refinement and delay development of VSV-based vaccines.

#### 6.2.4. Other vectored filovirus vaccine candidates

There are other vector approaches that have been evaluated for filovirus vaccines. A vaccinia-derived vector expressing ZEBOV GP protected guinea pigs but not cynomolgus

macaques despite induction of neutralizing antibody [77]. That failure, the concern of pre-existing immunity to vaccinia and the appearance of other more successful vaccine candidates, led to abandonment of the vaccinia approach. More recently, Bukreyev et al. [100] described the use of an intranasally administered paramyxovirus as a vector expressing ZEBOV GP to fully protect guinea pigs against parenteral ZEBOV challenge. The paramyxovirus constructs were immunogenic and provided complete protection against challenge. However, the significant safety concerns regarding the use of a live, non-attenuated human pathogen as a vector raise obvious concerns about its use as a human vaccine. The authors do mention the potential to use a paramyxovirus vector that infects other hosts but is attenuated in humans; however, the guinea pig studies would have to be repeated and safety issues would remain paramount.

### 7. Virus-like particles (VLP)

VLP are an attractive alternative to ‘traditional’ subunit vaccines in that they are an aggregate of viral proteins in native conformation without the safety concerns that attend attenuated or replication-deficient viruses. Co-expression of ZEBOV GP and VP40 in 293T cells resulted in the production of ZEBOV VLPs (eVLPs) that were indistinguishable by electron microscopy from live ZEBOV particles [15,101]. When eVLPs were cultured with mouse bone-marrow-derived dendritic cells, they induced maturation of the cells and production of inflammatory cytokines [102]. eVLPs were immunogenic in mice when given i.m. or i.p. and vaccinated mice were fully protected against challenge with mouse-adapted ZEBOV [57]. Further study in mice suggests that a Th1-type immune response with CD8<sup>+</sup> T lymphocytes is critical to the protection against ZEBOV challenge [55].

MARV VLPs (mVLPs) were generated in a similar fashion to the eVLPs and combined with RIBI adjuvant to vaccinate guinea pigs [103]. The plaque-reducing neutralization titer to neutralize 80% of the virus in vitro (PRNT-80) was 1:100 in mVLP-vaccinated guinea pigs; however, inactivated MARV-vaccinated guinea pigs had a PRNT-80 of 1:300. Guinea pigs from both the mVLP and inactivated MARV groups were fully protected against challenge with homologous guinea pig-adapted MARV. Hybrid VLPs containing the GP of ZEBOV and VP40 of MARV (or the VP40 of ZEBOV and GP of MARV) were only able to protect if the GP was to the homologous virus [16]. Guinea pigs vaccinated with a combination of mVLP and eVLP were fully protected against challenge with either ZEBOV or MARV, demonstrating that a pan-filovirus vaccine is possible. Results from NHP studies have not yet been reported nor have there been any attempts to optimize the dose and schedule of VLP vaccination. Production of sufficient VLP may be a concern especially if the dose required is high. In addition, results demonstrated efficacy in the more stringent NHP models are needed to advance VLP to the same level as other existing candidates.



## 8. Immunological correlates and mechanisms

To license vaccines using the FDA's Animal Rule requires demonstrating an understanding of the immunological mechanisms responsible for that protection. The response in humans to the vaccine must be sufficiently similar to the response in protected animals so that one can infer that protection in the animal will predict protection in the human.

For filoviruses, this challenge is even more daunting than it may first appear. Much of what we know about the immune system is based on studies in mice and humans. In recent years, our understanding of NHP immunology has improved, as NHP, particularly rhesus macaques, have been studied as surrogate models for HIV infection using simian immunodeficiency virus [104,105]. But there are many differences between human and NHP immune systems. Macaques have only three subtypes of IgG, lacking IgG<sub>3</sub> [106]. Many monoclonal antibodies that react to human lymphocytes are cross-reactive with lymphocytes from rhesus macaques, cynomolgus macaques, and African green monkeys, but many others are not. Multi-color flow cytometry has demonstrated that the patterns of expression for many markers on leukocytes are different from what has been observed in humans. For example, natural killer (NK) lymphocytes in rhesus macaques, cynomolgus macaques, and African green monkeys express CD8, whereas NK lymphocytes in humans typically do not [107,108] (D. Reed, personal observations). African green monkeys have a CD4:CD8 T lymphocyte ratio that is skewed towards CD8 T lymphocytes rather than the 2:1 ratio seen in humans [109,110]. What (if any) impact these differences have on the immunological response is currently unknown.

The majority of vaccine studies with ZEBOV and MARV have focused on GP. Hevey et al. [78] found that while GP alone conferred complete protection against MARV. NP alone did not. VRP expressing VP24, VP30, or VP40 protected BALB/c mice against ZEBOV challenge while only VP35 was protective in C57 BL/6 mice [54]. Studies with eVLP and mVLP have shown that while VP40 is essential for the structure of the VLP, VP40 alone is not sufficient to protect guinea pigs against a homologous virus [16]. One common theme that runs through these studies is that the potency of the immunological response to the vaccine is critical and that optimization of dose, route and schedule will likely be required for protection in NHP, particularly for challenge with ZEBOV or heterologous MARV.

Of the two 'arms' of the adaptive immune response, humoral immunity is the most easily measured, and passive transfer of sera can be used to demonstrate antibody-mediated protection. In the 1995 outbreak of ZEBOV in Kikwit, blood transfusions from convalescent patients appeared to protect sick patients, suggesting that humoral immunity could protect [111] although these findings are controversial and the number of patients was limited. Studies using monoclonal antibodies and immune serum in cell culture and in animals have also suggested that antibody can protect against ZEBOV

[18,20,53,78,112,113]. Hyper-immune equine sera, however, was unable to protect cynomolgus macaques from ZEBOV, although under some circumstances, it did delay viremia and time to death [114]. Antibody-mediated enhancement of infection in cell culture has been reported for VSV expressing ZEBOV GP [115]; however, other laboratories have not observed this effect [116]. Antibody capable of neutralizing ZEBOV or MARV in vitro has been difficult to measure before challenge of vaccinated animals and has not correlated with protection in NHP. Humoral immunity is clearly important for protection against filovirus infection but more study is needed to identify the critical parameters (concentration, affinity/avidity, IgG subtype) essential for good protection.

Identifying the cellular mechanisms that protect against infection with EBOV or MARV will be far more difficult. Memory T lymphocytes specific to a particular antigenic epitope comprise only a very small portion of T lymphocytes in a normal host. MHC tetramers will not be useful in clinical trials in humans or pivotal efficacy studies in outbred animals. Both intracellular cytokine staining (ICS) and ELISPOT have been used to detect memory T lymphocytes in humans and NHP, although debate continues on which assay is the more sensitive or reproducible. A series of recent publications have demonstrated how the use of peptide pools can be used to identify important epitopes for T-lymphocyte responses, even in outbred animals. Applying this improvement to traditional ELISPOT and ICS assays will help immensely in comparing responses between humans and animal models. Similarly, an ELISPOT assay to detect memory B lymphocytes has been described and has shown utility in detecting memory in smallpox vaccine recipients years after vaccination [117]. This assay could detect the frequency of cells capable of producing specific antibody after antibody titers in the blood have waned.

Nevertheless, in mice, CD8<sup>+</sup> T lymphocytes are important for protection against ZEBOV [55,58]. NHP studies with the adenovirus and VSV-vectored vaccines have also found the presence of CD8<sup>+</sup> T lymphocytes in vaccinated macaques by ICS before challenge [87,96]. Definitive data demonstrating the importance of CD8<sup>+</sup> T lymphocytes as a mechanism of protection in a relevant animal model, however, have not yet been reported.

## 9. Conclusions and the path forward

A decade ago, there seemed little hope for a vaccine that would protect against filoviruses. Equally troubling were the revelations that the former Soviet Union had 'weaponized' MARV and possibly ZEBOV [13]. Since that time, several vaccine candidates have been generated by using modern technology and have shown immense promise by protecting animals, particularly NHP, against challenge with MARV and ZEBOV. If nothing else, these animal studies have demonstrated that a vaccine is certainly now possible for both MARV and EBOV. A summary of the various vaccine efforts,

Table 1  
Summary of filovirus vaccine efforts

Approach	Antigen	Immunity	Efficacy		Concerns	Refs.
			Rodent	NHP		
Classical						
Killed	Whole virus	IgG+, low NT	Varies	Failed	Potency, schedule, adjuvant, safety	[75,103,118]
Attenuated	Whole virus	IgG+	Failed	Failed	Safety	[64]
Subunit						
Baculovirus	GP, GPΔTM	IgG+	Partial	ND	Potency, glycosylation	[60,78,79]
VLP	GP + VP40	IgG+, NT+, CD4+	Good	ND	Potency, schedule	[15,16,55,57,103]
DNA						
Alone	GP, NP	IgG+	Poor	ND	Potency, schedule	[79,80,119]
Prime/boost, w/Adv	GP	IgG+, no NT, CD4+	Good	Good	Schedule, potency	[86]
Vectored						
Vaccinia	GP, VP24	IgG+, low NT		Poor	Anti-vector immunity, safety, potency	[77]
VRP	GP, GP + NP, NP, VP35, VP30, VP24	IgG+, no NT, CTL, CD4+	Good	Good	Anti-vector immunity, potency, schedule, heterologous viruses	[19,54,58,60]
Adenovirus	GP, GP + NP	IgG+, CD8+	Good	Good	Anti-vector immunity, potency	[87]
VSV	GP	IgG+, no NT, no CMI	Good	Good	Safety	[96]
Parainfluenza	GP, GP + NP	IgG+	Good		Safety	[100]

the antigens examined, their efficacy and concerns is shown in Table 1. The road to licensure is long, however, and there are a number of scientific hurdles left for all of these candidates before they can be used in humans with a reasonable expectation that they would be safe, immunogenic, and efficacious. Along the road to licensure there will likely be considerable discussion about who should be vaccinated. Licensure of the general public is feasible but likely to meet resistance, particularly in western countries where the likelihood of filovirus infection is very low. Stockpiles of vaccine could be kept in case of a natural outbreak or a bioterrorist attack and distributed as needed. The recent report that VSV-vectored MARV vaccine can protect even when given postexposure offers some hope that these vaccines might be given postexposure as can be done with the live smallpox vaccine; we would caution that optimism by pointing out that in that report the vaccine was given immediately postexposure, that would be unlikely in either a natural outbreak or bioterrorist scenario. It seems likely though that a ring-type vaccination of surrounding areas would still be advisable as this would prevent secondary transmission. Under this type of scenario, development of therapeutics for filovirus infection is also needed to treat primary cases. Similar to what has been done for rabies control, filovirus vaccines might also be used in wild chimpanzees and gorillas in an effort to control outbreaks since there is evidence that outbreaks in these species may precede human cases and in some outbreaks initial human cases may have come from eating or handling infected animals [11,12]. This review has covered the most promising candidates, covering the advantages of each and where potential pitfalls may lie. It is hoped that the insights provided here on these efforts may aid efforts on developing vaccines for other emerging diseases or bioweapon threats.

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